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(56) Documents cited

**EP A1 0158420 EP A2 0119476**

**Biochemistry (U.S.) (1985) 24 p 4131-41**

**Cancer Res. (1985) vol 45 p 2421-4**

**Conference-Biol Interferon Syst. Proc. TNO-ISIR Meet.**

**Interferon Syst. 4th (86) p 167-71. C.A. 105(21)189118**

**Serond Symp. Publ. Raven Press (1985) p 237-42 vol 24**

**C.A. 104 (07) 049674**

**Conference NK Cells Other Nat. Eff. Cells (1982) p**

**355-60 C.A. 98 (07) 51589**

**J General Virology 65 p 629-33**

**Antiviral Research (Nether) (1984) vol 4 p 74**

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(54) **Antibodies to interferon**

(57) A monoclonal antibody to IFN which inhibits the cell-binding capability of the IFN or which inhibits the cytotoxicity-boosting activity of the IFN mediated by NK cells can be obtained by raising anti-IFN monoclonal antibodies by techniques known *per se* and screening them for the desired inhibitory activity, then culturing the cell line which produces the selected monoclonal antibody.

The IFN may be IFN $\alpha$  or especially, recombinant IFN $\alpha$ -2.

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## SPECIFICATION

### Antibodies to Interferon

- 5 This invention relates to the identification of antibodies to interferon, especially those exhibiting pronounced inhibition of the antiviral and/or cytotoxicity-boosting activity by means of an identification of the binding site(s). 5

The following abbreviations are used herein:—

- |    |      |                                     |    |
|----|------|-------------------------------------|----|
| 10 | BSA  | —Bovin seroalbumin                  | 10 |
|    | MAb  | —Monoclonal antibody (ies)          |    |
|    | IFN  | —Interferon                         |    |
|    | Kd   | —Kilodalton                         |    |
|    | SDS  | —Sodium lauryl sulphate             |    |
| 15 | PAGE | —Polyacrylamide gel electrophoresis | 15 |
|    | PBS  | —Phosphate buffer saline pH 7.2     |    |
|    | NK   | —Natural killer.                    |    |

- 20 Human alpha interferons are a family of closely related proteins containing both conserved and non-conserved regions in their primary sequences (1–3). The aminoacid sequences of many IFN subtypes have been deduced from the nucleotide sequences of the corresponding genes (4–5). Several recombinant and hybrid IFN's genes have been cloned, expressed in bacteria, their encoded products purified to homogeneity, and characterized (5–9). 20

- 25 Recombinant IFN molecules, similarly to the natural species, exhibit biological functions such as the antiviral and antiproliferative activities (5,8,10). IFNs also display several immunomodulating activities as the ability to augment the activity of NK cells (11–12). The study of the biological effects mediated by different recombinant and hybrid IFN molecules has allowed the identification of a IFN subtype displaying strong antiviral and antiproliferative activities but lacking the ability to boost the NK cytotoxicity 13. This might suggest the existence of distinct functional domains on IFN responsible for its different biological activities. 25 30

- Structure-function relation studies on the IFN $\alpha$ -2 molecule have been reported by several groups using different approaches. Chemical and enzymatic modifications of the molecular have been used to examine the role of certain aminoacid residues and the disulphide bridges on the biological function of IFN (14–16). On the other hand, the function of predetermined IFN variant molecules lacking segments of their primary sequences constructed by recombinant DNA technology has also been studied (9,17). 30 35

- An alternative approach, that is being used to monitor functional sites of biologically active molecules, is the immunochemical mapping by monoclonal antibodies. Several groups have reported the generation of hybridomas secreting monoclonal antibodies specific for IFN $\alpha$ -2 (18–23). They have been primarily used as reagents for the purification and assay of interferons (24–26). In recent studies, the anti-IFN MAB with neutralizing and non-neutralizing capabilities have been used as tools to investigate structure-function relationships (22–23). In one of these studies, the use of a panel of MAB in combination with IFN peptides have allowed the mapping, at the primary structure level, of antigenic determinants related to the IFN antiviral activity (23). 35 40

- 45 We have now found that there are at least three different binding sites or epitopes and that by selection of MABs with certain binding characteristics enables highly active MABs to be identified. 45

- According to the present invention we provide a monoclonal antibody to IFN which inhibits the cell-binding capability of the IFN or which inhibits the cytotoxicity-boosting activity of the IFN mediated by NK cells. 50

There is also provided a method for preparing such antibodies by raising anti-IFN monoclonal antibodies by techniques known *per se* and screening them for the desired inhibitory activity, then culturing the cell line which produces the selected monoclonal antibody.

- 20 Twenty different monoclonal antibodies directed to rIFN $\alpha$ -2 have been obtained by immunobinding techniques and used to define distinct epitopes on the IFN molecule. The functional relation of these epitopes has been studied by three different biological functions, namely, the antiviral activity, the boosting of NK-mediated cytotoxicity and the IFN binding to its cellular receptor. 55

- The following description provides an Example of the method according to the present invention. 60

### MATERIALS AND METHODS

#### Interferon

- The IFN $\alpha$ -2 was purified by immunoaffinity chromatography on an anti-IFN $\alpha$ -2 NK-2 MAB-Sepharose (Celltech, UK) column from lysates of *Escherichia coli* which expresses the human 65

IFN $\alpha$ -2 gene contained in an inserted plasmid (Antibioticos S.A., Madrid, Spain).

SDS-PAGE analysis of purified IFN preparation revealed the presence of a major 20 Kd band (>90% of total protein), corresponding to the IFN molecule. This preparation was utilized for immunization and screening of monoclonal antibodies.

5

#### *Immunization and hybridoma production*

Balb/c female mice (Iffa Credo, Lyon, France) were injected intraperitoneally with 10  $\mu$ g of purified rIFN $\alpha$ -2 in complete Freund's adjuvant on day -28, and with 25  $\mu$ g in incomplete Freund's adjuvant on day -18. On day -3, mice were boosted intravenously with 25  $\mu$ g of IFN $\alpha$ -2 in 200  $\mu$ l of phosphate buffer saline (PBS). On day 0, P3 $\times$ 63Ag.8.6.5.3 and SP2 mouse myeloma cells were fused with spleen cells from the immunized mice, using 50% (w/w) poly-ethyleneglycol as described (27). Three different fusions were performed (CYS1, CYS2 and CYS3). Cells were aliquoted into ten 96-well plates (Costar, Cambridge MA) and grown in selective HAT medium as previously described (27,28).

5

15 After two weeks, culture supernatants from wells displaying hybridoma growth were harvested and screened for specific binding to rIFN $\alpha$ -2. A number of 960 different hybridoma cultures were screened for their abilities to secrete antibodies directed to IFN in an indirect binding assay using <sup>125</sup>I-labeled IFN $\alpha$ -2. Those hybridomas found positive were cloned in soft agar. Selection of active clones was carried out using the indirect binding assay.

15

20 Immunoglobulin subclass of the anti-IFN CYS MAb was determined by double immunodiffusion with anti-mouse subclass specific antibodies (Nordic Lab., Netherlands). Purification of anti-IFN CYS MAb from mouse ascites fluids was carried out by immunoabsorption on a column of *As. aureus*-protein A coupled to Sepharose as described (29).

20

#### *25 Radiolabeling, immunoprecipitation and electrophoresis*

25

Purified rat anti-mouse Kappa chain 187-1 MAb (20  $\mu$ g) and rIFN $\alpha$ -2 (10  $\mu$ g) were radioiodinated in solution with chloroglycoluril (30). For immunoprecipitation, equal amounts of input radioactivity of <sup>125</sup>I-labelled purified rIFN $\alpha$ -2 were mixed with 75  $\mu$ l of MAb-containing culture supernatants.

30 To isolate immune complexes, 30  $\mu$ l of purified 187.1 rat anti-mouse Kappa chain MAb coupled to Sepharose (1mg/ml) were added. Immunoprecipitates were processed and the samples subjected to SDS-15% PAGE and autoradiography with enhancing screens as previously described (31).

30

35 *Indirect binding assays* Initial screening of anti-IFN producing hybridomas used softwell polyvinylchloride 96 microtiter plates (MIC-2000 Dynatech Lab. Inc.) coated with 50 $\mu$ l of purified 187.1 rat anti-mouse Kappa chain MAb (10 $\mu$ g/ml) (32) for 1h at 37°C. Plates were washed twice with 1%BSA in PBS. Next, hybridoma culture supernatants (50 $\mu$ l/well) were added for 45 min. Then, the plates were washed three times with 0.25% BSA in PBS. In the second step, <sup>125</sup>I-labeled rIFN $\alpha$ -2 (100.000 cpm/well) was added and incubated for 45 min. Plates were washed three times with 0.25% BSA in PBS, dried and the radioactivity estimated in a gamma counter. All assays were performed in duplicate.

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40 A modified indirect binding assay was also used for anti-IFN CYS MAb. Soft-well plates were coated with purified human rIFN $\alpha$ -2 and incubated with CYS MAb for 45 min at room temperature followed by a second incubation with the <sup>125</sup>I-labeled 187.1 anti-mouse Kappa chain MAb (50.000 cpm/well).

45

#### *Competitive binding assays*

50 A simple method devised for analyses of epitopes defined by CYS MAb on the IFN molecule is briefly described. Hybridoma culture supernatants (50 $\mu$ l/well) containing anti-IFN CYS MAb were added to soft-well polyvinylchloride 96-microtiter plates coated with purified 187.1 anti-mouse Kappa chain MAb. Incubation was allowed for 1 h at room temperature, and plates were washed three times with 0.25% BSA in PBS.

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55 Next, <sup>125</sup>I-labeled IFN $\alpha$ -2 samples (80-100.000 cpm/well), which have been previously incubated in a separate plate for 1 h at 4°C with 50 $\mu$ l of culture supernatants containing each of the different twenty anti-IFN CYS MAb, were added to the anti-IFN $\alpha$ -2 MAb-187.1 coated wells. Plates were incubated for 30 min at room temperature, washed three times with 0.25% BSA in PBS, dried, and the radioactivity estimated in a gamma counter. Identical competition experiments were carried out with different combination of the different anti-IFN CYS MAb.

55

60 A modified competition assay was performed to investigate the relationship between epitopes defined by anti-IFN CYS MAb and the one recognized by the commercially available anti-IFN $\alpha$ -2 NK-2 MAb. In this assay, <sup>125</sup>I-labeled IFN samples were incubated with 50  $\mu$ l of culture supernatants of the twenty different CYS MAb for 1 h at 4°C. in separate Eppendorf tubes. Then, 20  $\mu$ l of a 1:1 slurry containing 10  $\mu$ l of NK-2 MAb Sepharose or 10  $\mu$ l of IgG-Sepharose from an irrelevant mouse MAb were added to each tube and shaken for 1 h at 4°C. Samples were

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washed five times with 0.25% BSA in PBS and the radioactivity bound to the beads estimated.

#### Cell binding assays

The  $^{125}\text{I}$ -labeled IFN preparation used for binding to human cells was previously purified by absorption to a microcolumn of anti-IFN CYS 3/22 MAb coupled to Sepharose (300  $\mu\text{l}$ ) followed by low pH elution with 0.1M glycine pH 2.5; and neutralization with 1M Tris-HCl pH8.0. Competition cell binding assays were performed by incubation of  $^{125}\text{I}$ -labeled IFN with anti-CYS MAb for 1 h at 4°C. Then, the IFN samples were added to microtiter plates containing 10  $\mu\text{l}$  of a cell suspension of  $2.5 \times 10^7$  cells/ml of the U-937 human myelomonocytic line, and shaken for 45 min at 4°C. Cells were washed three times with 0.25% BSA in PBS, pellets were removed with two washes of 0.25% BSA in PBS and radioactivity estimated in a gamma counter. All assays were performed in duplicate.

#### Functional assays

The antiviral activity of IFN was tested basically as described (33). Hela cells were grown in 96 wells microtiter plates in DME/10% FCS until they reached confluency. Then, the cells were washed and incubated with known amounts of IFN in the presence of anti-IFN MAb for 16 hr at 37°C. After this incubation, the cells were washed and vesicular stomatitis virus (VSV) (0.01 pfu/cell) was added in DME/2% FCS. After 24 hr of incubation at 37°C, the protection by IFN of the cytopathic effect caused by VSV was evaluated using a hexosaminidase assay (34). Briefly, the cells were washed 3 times with PBS before 60  $\mu\text{l}$  of a substrate solution (p-nitrophenyl-N-acetyl-D-glucosaminide) of the lysosomal hexosaminidase was added to each well and incubated for 1 h at 37°C. The reaction was stopped by adding 90  $\mu\text{l}$  of Tris-glycine buffer pH 10.2 containing 5 mM EDTA and the absorbance at 405 nm was measured.

In the NK-mediated cytotoxic assays, human mononuclear cells isolated from the venous blood of normal volunteers by Ficoll-Isopaque gradient centrifugation were used as effector cells. To test the effect of the different MAb in the IFN boosting of the NK activity,  $1-2 \times 10^3$  units of IFN in 0.1 ml were incubated either alone or in the presence of different MAb for 30 min. Then, effector cells were added and two hours later the cells were washed twice. Control effector cells incubated with medium alone or irrelevant MAb were also included.

The effector cells were tested for cytotoxic activity at 25:1 effector to target cells ratio against  $5 \times 10^3$   $^{51}\text{Cr}$ -labeled K-562 target cells during 3 hr as previously described (35).

The inhibition of the interferon boosting of the NK activity by different MAb was calculated according to the formula:

$$\% \text{ of Inhibition} = 100 \times \frac{\text{Cytotoxicity with IFN} - \text{Cytotoxicity with IFN and MAb}}{\text{Cytotoxicity with IFN} - \text{Cytotoxicity without IFN}}$$

## RESULTS

#### Production and specificity of anti-IFN CYS MAb

To generate monoclonal antibodies to human IFN $\alpha$ -2, mice were immunized with a highly purified preparation of recombinant IFN $\alpha$ -2. Hybrids were selected by their ability to secrete antibodies that bind  $^{125}\text{I}$ -labeled purified IFN $\alpha$ -2 in a solid-phase radioimmunoassay, and the positive wells were also tested by an inverse radioimmunoassay.

Twenty different hybridoma lines secreting antibodies were found positive in both assays and then cloned (Table I). The different CYS MAb gave similar levels of binding of  $^{125}\text{I}$ -labeled IFN $\alpha$ -2 with the exception of CYS 3/4 that showed lower binding. In the inverse radio-immunoassay, MAb CYS 3/4 and CYS 3/12 gave higher binding than other CYS MAb.

The specificity of CYS MAb for the IFN molecule was confirmed by immunoprecipitation analysis from  $^{125}\text{I}$ -labeled IFN preparations. All the MAb previously selected specifically precipitated a 20 Kd molecule with identical electrophoretic mobility as the one recognized by the control anti-IFN $\alpha$ -2 NK-2 MAb. The intensity of the band precipitated by the CYS 3/4 MAb was weaker than the obtained with the other anti-IFN CYS MAb.

#### Epitopes on the rIFN $\alpha$ -2 defined by CYS MAb

The epitopes defined by CYS MAb were studied by cross-competition binding experiments. First, we determined the relative position of the antigenic determinants recognized by CYS MAb respect the one defined by the anti-IFN NK-2 MAb (Table II). Six different CYS MAb (CYS 1/3, CYS 1/4, CYS 1/5, CYS 3/8, CYS 3/12 and CYS 3/14) strongly inhibited the binding of  $^{125}\text{I}$ -labelled IFN to NK-2 MAb coupled to Sepharose, suggesting that they recognize an identical or overlapping epitope as the one defined by NK-2 MAb, hereafter designated as site A. Furthermore, binding of labeled IFN to these anti-NK-2-like CYS MAb, was completely competed by each other (data not shown). The CYS 1/3 MAb was included as representative of the group of CYS MAb directed to the site A of IFN.

Cross-competition experiments in the binding of  $^{125}\text{I}$ -labeled IFN with other anti-IFN MAb recognizing epitope (s) distinct to the site A, are summarized in Table III.

Two distinct additional epitopes were defined by different CYS MAb. A group of seven MAb (CYS 3/2, CYS 3/7, CYS 3/18, CYS 3/21, CYS 3/22, CYS 4/4 and CYS 4/7) recognized identical or overlapping epitopes, designated as site B. Moreover, site C was defined by only one MAb, CYS 3/19. The MAb most representative of the epitopes A, B and C (CYS 1/3, CYS 3/22 and CYS 3/19, respectively) did not significantly interfere with each others' binding. Similar results were obtained by competitive binding using radiolabeled anti-IFN MAb. In addition, partial overlapping sites were also defined by several MAb. 1) The MAb CYS 3/15, that partially competed the binding of MAb representative for sites A and B, and completely abolished binding of the MAb specific for site C. 2) MAb CYS 3/10, CYS 3/17, CYS 3/20 and CYS 4.5, that partially competed the binding of MAb for sites B and C.

Results obtained in the competition experiments with the CYS 3/4 MAb were not included in Table III because of its weak binding to soluble radiolabeled IFN. This MAb did not affect binding of  $^{125}\text{I}$ -labeled IFN to other CYS MAb (data not shown). These results, together with binding studies suggested that the epitope recognized by this MAb might be dependent of the different conformation of the IFN molecule in soluble or solid-phase-coupled states.

#### *Analysis of the functional sites on IFN $\alpha$ -2 by using CYS MAb*

To determine whether the CYS MAb directed to epitopes localized on different regions might affect differentially the function of the IFN molecule, the antibodies were tested either in the antiviral activity, or the boosting of the cytotoxicity mediated by NK cells.

The neutralization of the antiviral activity was carried out by using increasing doses of IFN and a constant amount of the anti-IFN CYS MAb representatives for sites A, B and C. Results obtained with the twenty different anti-IFN MAb are summarized in Table IV. The group of seven MAb directed to the site B, with the exception of CYS 3/18, strongly blocked the antiviral activity of IFN $\alpha$ -2, with a neutralizing activity between 1.200 and 1.400 Units/ml of IFN $\alpha$ -2. Comparative studies with the group of six MAb specific for site A demonstrated a lower inhibition of the antiviral activity, neutralizing from 350 to 500 Units/ml of IFN. Conversely, the MAb defining the site C did not interfere significantly with the antiviral activity of IFN. Those anti-IFN MAb recognizing overlapping epitopes showed different effects on the antiviral activity, probably dependent on their degree of proximity to the antigenic site B.

The effects of the anti-IFN CYS MAb on the enhancing effect of IFN $\alpha$ -2 on the cell cytotoxicity mediated by NK cells are shown in Table IV. MAb directed to site B abolished the augmentative effect of IFN on the killing activity, whereas MAb directed to site A exerted very small inhibitory effects. The MAb directed to site C caused a partial blockade of this IFN function. These results were consistently reproduced in four different experiments.

These results suggest that the epitope B is localized close, or overlapping, to an active functional site of IFN $\alpha$ -2; whereas epitopes A and C appear to play less important functional roles on the IFN molecule.

#### *Interaction of epitopes A, B and C with the IFN $\alpha$ -2 cellular receptor*

To examine the possible relationship between the IFN $\alpha$ -2 A, B and C epitopes and the IFN interacting site (s) with the cellular receptor, we carried out competition binding experiments using the human myelomonocytic cell line U-937 which bears high number of receptors for IFN.

The  $^{125}\text{I}$ -labeled IFN preparation used in the cell-binding experiments was further purified, after iodination by immunoabsorption to an anti-IFN $\alpha$ -2 column of CYS 3/22 MAb coupled to Sepharose, to allow the selection of labeled IFN molecules maintaining intact the functionally important site B. This IFN preparation gave comparable binding to immobilized CYS MAb specific for the three distinct epitopes, demonstrating that, in addition to the site B, it also preserved the sites A and C (Table V). In addition, the binding of radiolabeled IFN to U-937 cells could be completely competed out in a dose-dependent fashion by unlabeled IFN, demonstrating the specificity of this interaction (data not shown). The specific binding of  $^{125}\text{I}$ -labeled IFN $\alpha$ -2 was completely abolished by preincubation with anti-IFN MAb directed to site B, the one strongly associated with different functions. The MAb specific for site C partially affected this binding. Conversely, very little inhibition on the IFN binding was exerted by MAb specific for site A (Table VI).

These results suggested that the antigenic site B maps close to or overlapping with the IFN cell membrane receptor. This would explain the strong blockade effect exerted by anti-IFN $\alpha$ -2 B MAb on different functional activities and the weak inhibitory effect of anti-IFN MAb directed to site A.

Table I.- Binding of CYS MAb to human rIFN $\alpha$ -2.

	<u>MAB</u>	<u>Is type</u>	<u>Binding of <sup>125</sup>I-IFN</u>	<u>Binding of <sup>125</sup>I-187</u>	
5			<sup>a</sup> Bound Radioactivity (cpm)		5
	CYS 1/3	IgG1	3195	4593	
10	CYS 1/4	IgG1	2941	2590	10
	CYS 1/5	IgG1	3349	5742	
	CYS 3/2	IgG1	2808	4361	
15	CYS 3/4	IgG2a	700	19467	15
	CYS 3/7	IgG1	3561	4014	
20	CYS 3/8	N.D.	2356	2493	20
	CYS 3/10	IgG1	3077	3938	
	CYS 3/12	IgG1	3506	19101	
25	CYS 3/14	IgG1	3025	3206	25
	CYS 3/15	IgG1	2670	3193	
30	CYS 3/17	IgG1	2568	8281	30
	CYS 3/18	IgG1	2816	3270	
	CYS 3/19	IgG1	3757	2956	
35	CYS 3/20	IgG2a	2308	2956	35
	CYS 3/21	IgG1	2566	3097	
40	CYS 3/22	IgG1	3858	4819	40
	CYS 4/4	IgG1	2440	2976	
	CYS 4/5	IgG1	2621	4659	
45	CYS 4/7	IgG1	2940	5943	45
	X63 (Negative Control)	IgG1	358	1714	
50	<sup>a</sup> Binding of <sup>125</sup> I-labeled IFN or <sup>125</sup> I-labeled anti-mouse kappa chain 187.1 MAB to the anti-IFN mouse MAB, specifically complexed to purified 187.1 or IFN-coated polivinyl microtiter wells, respectively, was determined in the indirect				50
55	binding assay. Nonspecific binding was determined by using the P3X63 mouse myeloma culture supernatant, that contains standard concentration of mouse $\gamma$ 1,k chains.				55

Table II.- Competition of the binding of  $^{125}\text{I}$ -IFN to NK2-Sepharose by CYS MAb.

5	<u>MAb</u>	<u>Binding <math>^{125}\text{I}</math>-IFN (cpm)</u>	<u><sup>a</sup> Inhibition (%)</u>	5
	CYS 1/3	569	95	
10	CYS 1/4	783	86	10
	CYS 1/5	773	86	
	CYS 3/2	3912	0	
15	CYS 3/4	2499	0	15
	CYS 3/7	2651	0	
	CYS 3/8	1312	64	
20	CYS 3/10	2612	0	20
	CYS 3/12	695	89	
25	CYS 3/14	440	100	25
	CYS 3/15	2717	0	
	CYS 3/17	2836	0	
30	CYS 3/18	3818	0	30
	CYS 3/19	2439	0	
35	CYS 3/20	2437	0	35
	CYS 3/21	2667	0	
	CYS 3/22	4078	0	
40	CYS 4/4	3832	0	40
	CYS 4/5	3366	0	
45	CYS 4/7	2187	28	45
	X63 (Negative Control)	2435	0	

50

<sup>a</sup> No inhibition and 100% inhibition were determined with mouse myeloma P3X63 culture supernatant, and the CYS 3/14 MAb, giving strongest competition, respectively.

50

Table III.- Epitopes on the rIFN $\alpha$ -2 defined by CYS MAb.

Immobilized MAb	Soluble MAb Competitor														
	X63	CYS 1/3	CYS 3/2	CYS 3/7	CYS 3/10	CYS 3/15	CYS 3/17	CYS 3/18	CYS 3/19	CYS 3/20	CYS 3/21	CYS 3/22	CYS 4/4	CYS 4/5	CYS 4/7
						a Inhibition (%)									
CYS 1/3	0	100	10	13	3	49	36	30	4	42	31	9	30	36	20
CYS 3/2	0	35	100	97	12	58	45	90	0	51	98	96	97	44	99
CYS 3/7	0	21	100	100	12	46	21	90	0	29	100	99	96	48	100
CYS 3/10	0	35	50	0	100	96	93	9	70	88	31	38	52	98	5
CYS 3/15	0	39	0	0	95	100	93	0	92	93	0	9	0	95	0
CYS 3/17	0	22	5	0	90	98	100	6	92	84	18	14	22	98	0
CYS 3/18	0	1	99	100	0	14	1	100	0	0	100	100	100	94	100
CYS 3/19	0	27	40	10	100	100	100	7	100	100	17	18	36	100	19
CYS 3/20	0	10	0	0	100	100	100	1	96	100	37	0	11	96	8
CYS 3/21	0	15	100	100	0	4	30	99	10	25	100	100	100	97	100
CYS 3/22	0	21	100	96	16	47	39	85	6	46	99	100	93	21	100
CYS 4/4	0	0	99	97	0	5	0	98	0	0	97	95	100	90	100
CYS 4/5	0	29	72	81	97	96	98	77	59	96	92	77	83	100	96
CYS 4/7	0	44	92	90	15	47	25	84	0	30	98	96	94	87	100

<sup>a</sup> No inhibition and 100% inhibition were determined with mouse myeloma P3X63 culture supernatant and the homologous MAb competitor, respectively, in the competitive binding assay, using anti-IFN MAb complexed to purified anti-mouse Kappa chain MAb coated to polyvinylchloride wells and <sup>125</sup>I-labeled rIFN $\alpha$ -2 as described in Material and Methods.



Table IV.- Inhibition of antiviral and boosted NK activities of rIFN $\alpha$ -2 by MAb.

5	<sup>a</sup> Antiviral activity				<sup>b</sup> IFN boosted NK cytotoxicity		5
	Neutralization Inhibition		Specific release	Inhibition of the boosting effect			
	MAb added	Epitope	(U/ml)	(%)	(%)	(%)	
10	X63	---	0	0	60	0	10
15	CYS 1/3	A	400	31	56	15	15
	CYS 1/4	A	400	28	53	25	
	CYS 1/5	A	400	35	51	33	
	CYS 3/8	A	500	41	55	19	
20	CYS 3/12	A	400	33	50	37	20
25	CYS 3/14	A	350	29	55	17	25
	CYS 3/2	B	1400	82	38	85	
	CYS 3/7	B	1200	68	45	55	
	CYS 3/18	B	300	21	50	39	
30	CYS 3/21	B	1300	74	39	82	30
35	CYS 3/22	B	1400	78	37	89	35
	CYS 4/4	B	1200	73	42	70	
	CYS 4/7	B	1200	71	40	75	
	CYS 3/19	C	50	10	49	41	
40	CYS 3/10	B&C	500	44	37	88	40
45	CYS 3/17	B&C	350	26	50	40	45
	CYS 3/20	B&C	150	20	53	28	
	CYS 4/5	B&C	350	26	44	61	
50	CYS 3/15	A&B&C	350	25	51	33	50
	CYS 3/4		0	0	60	0	
							55

Table IV.-

<sup>a</sup> The amount of IFN neutralized was calculated from the shift of the 50% protection of the monolayer, in presence of equal amounts of the different monoclonal antibodies. The % inhibition of the activity of 1000 U/ml of IFN is also shown.

<sup>b</sup> Human peripheral mononuclear (NK) cells (E:T=25:1) were incubated for 2 hours with  $1-2 \times 10^3$  U of IFN $\alpha$ -2, previously treated with equal amounts of different anti-IFN MAb for 30 min. Then, <sup>51</sup>Cr-labeled target cells were added and the assay completed. The spontaneous <sup>51</sup>Cr release in the absence of NK cells was 11%. Specific <sup>51</sup>Cr release determined on effector cells incubated with P3X63 control antibody in the absence or in the presence of IFN was of 34% and 60%, respectively. No inhibition and 100% inhibition of the IFN boosting effect is expressed relative to cultures treated or non-treated with IFN. Data represent the average of four independent experiments.

Table V.-Binding of CYS MAb to <sup>125</sup>I-labeled IFN $\alpha$ -2 affinity-purified on a column of CYS 3/22-Sepharose.

Antigenic determinant	MAb	Binding of <sup>125</sup> I-IFN $\alpha$ -2 <sup>a</sup> Radioactivity bound (cpm)
A	CYS 1/3	2827
B	CYS 3/22	3833
C	CYS 3/19	3087
-	X63	259

<sup>a</sup>Binding of <sup>125</sup>I-IFN (20,000 cpm), further purified after labeling on a column of CYS 3/22 coupled to Sepharose, to the anti-IFN mouse MAb specifically complexed to purified 187.1-coated polivinyl microtiter wells, was determined in the indirect binding assay.

Table VI.-Effect of anti-IFN MAb on the binding of <sup>125</sup>I-labeled rIFN $\alpha$ -2 to the myelomonocytic human cell line U-937.

MAb	Epitope	Binding of <sup>125</sup> I-IFN <sup>a</sup> Radioactivity bound (cpm)
X63	Negative Control	3009
CYS 1/3	A	1885
CYS 3/12	A	2486
CYS 3/22	B	394
CYS 4/7	B	573
CYS 3/19	C	1248

<sup>a</sup>The amount of radiolabeled rIFN $\alpha$ -2 (input=30,000 cpm) bound to the U-937 cells was determined in the cell binding assay as described under Materials and Methods. The values are the average of two independent experiments.

Different antigenic sites on the human rIFN $\alpha$ -2 and related to the functional domains involved in its antiviral, immuno-modulating and cellular receptor-binding activities by using a panel of neutralizing anti-IFN MAb, the MAb described here were initially selected by binding of <sup>125</sup>I-labeled IFN $\alpha$ -2 in a solid-phase radioimmunoassay. The specificity of the MAb for IFN has been clearly demonstrated by immunoprecipitation of the 20 Kd IFN molecule. The CYS 3/4 MAb displayed binding characteristics different from the rest of the MAb. In fact, its reactivity for IFN coupled to a solid-phase was much higher than for IFN in solution. This MAb also recognized the IFN molecule blotted onto nitrocellulose paper after SDS-PAGE separation.

The remaining 19 anti-IFN CYS MAb defined three spatially separated epitopes (A, B and C sites) and two partially overlapping antigenic sites on the IFN molecule as demonstrated by cross-inhibition binding experiments. Nevertheless, the ability of one MAb to inhibit the binding of other MAb does not necessarily imply that both MAb are directed to the same epitope on the IFN molecule. It is likely that MAb which bind to neighboring sites will interfere each others binding. The future use of other epitope mapping criteria such as binding to either distinct IFN peptides (23) or to different IFN subtypes (22, 24) will probably result in the definition of a higher number of antigenic sites.

Sites A and B, but not site C, appear to be involved on the antiviral activity of IFN. However, quantitative differences have been distinguished between the neutralizing capabilities of the groups of MAb directed to site A and site B. The functional involvement of site A on this activity is in agreement with previous reports describing the neutralizing activity of the NK-2 MAb (18, 23), since our anti-IFN $\alpha$ -2 A MAb recognize an epitope (s) closely related to that defined by NK-2. The epitope recognized by NK-2 is a conformational determinant formed by

the contribution of the 15 N-terminal amino/acid residues together with the region between residues 60 to 111 (23).

The study of the functional involvement of epitopes A, B and C in other IFN-mediated biological effect, such as the enhancement of the NK-mediated cytotoxicity by IFN confirms the functional differences observed between sites A and B. The inhibition studies of this IFN-mediated activity by the different anti-IFN MAb suggested that the epitope B also appears to be implicated in this IFN function, whereas epitope A does not. Surprisingly, site C, which was not required in the antiviral activity, seems to be partially involved in this IFN function. These findings clearly demonstrate that distinct functional domains participate in the different IFN-mediated activities. Moreover, they suggest that topographically distinct IFN regions are responsible for its antiviral and the enhancing of NK activities. These observations are in good agreement with conclusions derived from the study of the antiviral and the immunomodulating activities of distinct hybrid IFN molecules (13).

The IFN's interact with IFN-sensitive cells through specific binding to high affinity receptors on the cell surface (36-37). The receptor molecules have been biochemically identified and characterized by chemical crosslinking using <sup>125</sup>I-labeled IFN as a probe (38-39). The number of receptor present on the cell membrane is very low and it varies in different cell types (36). The binding of IFN to its receptor is required for biological activity.

The functional significance of the inhibition exerted by MAb on the biological activity of IFN could have different explanations. For example, the MAb could hamper the IFN-cellular receptor binding by either direct steric or by indirect allosteric hindrance. Alternatively, MAb could induce conformational changes on IFN affecting its activity. We have demonstrated that MAb specific for site B completely inhibit binding of radiolabeled IFN to the human U-937 cell line. These findings strongly suggest that epitope B may be localized either within or in the proximity of the IFN-interacting site with the cellular receptor. That would explain the dramatic inhibitory effect exerted by anti-IFN MAb directed to site B on all different IFN functions tested. By the contrary, MAb to site A do not significantly alter binding of IFN to cells indicating that they inhibit the antiviral activity without affecting the IFN-cellular receptor interaction. Previous studies have suggested that MAb NK2 may block IFN functions by preventing binding of IFN to susceptible cells (40). However, our anti-IFN MAb specific for site A, which overlaps with the one recognized by NK-2 MAb, do not significantly inhibit this binding.

A striking finding is the partial blockade of IFN binding by anti-IFN $\alpha$ -2 C MAb. This MAb does not give any inhibition of the antiviral activity of IFN but it partially blocks the IFN enhancing effect of NK-mediated cytotoxicity. These results suggest that the site C is not relevant for the antiviral activity but it is near to other site associated with the immunomodulatory activity of IFN.

According to the invention, therefore, it is possible to identify antibodies to IFN which bind to sites other than site A and to select from them antibodies which have potent inhibitory activity against the cell-binding capability or the cytotoxicity-boosting activity of the IFN. If it is then desired to produce supplies of such an antibody, the parent cell-line can be identified and then cultured.

#### REFERENCES

- 1.-Rubinstein, M., W.P. Levy, J. Moschera, C.Y. Lai, R.D. Hershberg, R.T. Bartlett, and S. Pestka. 1981. "Human leukocyte interferon: isolation and characterization of several molecular forms". Arch. Biochem. Biophys. 210:307.
- 2.-Allen, G. and K.H. Fantes. 1980. "A family of structural genes for human lymphoblastoid (leukocyte-type) interferon". Nature 287:408.
- 3.-Todokoro, K., D. Kioussis, and C. Weissmann. 1984. "Two non-allelic human interferon alpha genes with identical coding regions". EMBO J. 3:1809.
- 4.-Levy, W.P., M. Rubinstein, J. Shively, V. Del Valle, C.Y. Lai, J. Moschera, L. Brink, L. Gerber, S. Stein, and S. Pestka. 1981. "Amino acid-sequence of a human leukocyte interferon". Proc. Natl. Acad. Sci. USA 78:6186.
- 5.-Pestka, S. 1983. "The human interferons—from protein purification and sequence to cloning and expression in bacteria: before, between and beyond". Arch. Biochem. Biophys. 221:1.
- 6.-Goeddel, D.V., D.W. Leung, T.J. Dull, M. Gross, R.M. Lawn, R. McCandliss, P.H. Seeburg, A. Ullrich, E. Yelverton, and P.W. Gray. 1981. "The structure of eight distinct cloned human leukocyte interferon cDNAs". Nature 290:20.
- 7.-Nagata, S., N. Mantei, and C. Weissmann. 1980. "Structure of one of eight or more distinct chromosomal genes for human interferon- $\alpha$ ". Nature 287:401.
- 8.-Rehberg, E., B. Kelder, E.G. Hoal, and S. Pestka. 1982. "Specific molecular activities of recombinant and hybrid leukocyte interferons". J. Bio. Chem. 257:11497.
- 9.-Streuli, M., A. Hall, W. Boll, W.E. Stewart II, S. Nagata, and C. Weissmann. 1981. "Target cell specificity of two species of human interferon  $\alpha$  produced in *Escherichia coli* and the hybrid molecules derived from them". Proc. Natl. Acad. Sci. USA 78:2848.
- 10.-Goeddel, D.V., E. Yelverton, A. Ullrich, H.L. Heyneker, G. Miozzari, W. Holmes, P.H. Seeburg,

- T.Dull, L.May, N.St bbing, R.Crea, S.Maeda, R.McCandliss, A.Sloma, J.M.Tabor, M.Gross, P.C.Familletti, and S.Pestka. 1980. "Human leukocyte interferon produced by *E.coli* is biologically active". *Nature* 287:411.
- 11.—Herberman,R.B. 1984. "Interferon and cytotoxic effector cells" in J.Vilcek and E. De  
5 Mayer (eds.), *Interferon*. Elsevier Science Publishers, Amsterdam. Vol.2, pp.61. 5
- 12.—Ortaldo,J.R., A.Mason, E.Rehberg, J.Moschera, B.Kelder, S.Pestka, and R.B. Herberman.  
1983. "Effects of recombinant and hybrid recombinant human leukocyte interferons on cytotoxic  
activity of natural killer cells". *J.Biol.Chem.* 258:15011.
- 13.—Ortaldo,J.R., R.B.Herberman, C.Harvey, P.Osheroff, Y—Ch.E.Pan, B.Kelder, and S. Pestka.  
10 1984. "A species of human  $\alpha$  interferon that lacks the ability to boost human natural killer 10  
activity". *Proc.Natl.Acad.Sci.USA* 81:4926.
- 14.—Streuli,M., S.Nagata, and C.Weissmann. 1980. "At least three human type  $\alpha$  interferons:  
structure of  $\alpha 2$ ". *Science* 209:1343.
- 15.—Morehead,H., P.Johnston, and R.Wetzel. 1984. "Roles of the 29—138 disulfide bound of  
15 Subtype A of human  $\alpha$  interferon in its antiviral activity and conformational stability". *Biochemis- 15*  
*try* 23:2500.
- 16.—Ackerman,S.K., D.ZurNedden, M.Heintzelman, M.Hunkapiller, and K.Zoon. 1984. "Bio-logic  
activity in a fragment of recombinant human interferon  $\alpha'$ ". *Proc.Natl.Acad.Sci.USA* 81:1045.
- 17.—Chang,N.T., H—F.Kung, and S.Pestka. 1983. "Synthesis of a human leukocyte interferon  
20 with a modified carboxy terminus in *Escherichia coli*". *Arch.Biochem.Biophys.* 221:585. 20
- 16.—Secher,D.S., and D.C.Burke. 1980. "A monoclonal antibody for large-scale purification of  
human leukocyte interferon". *Nature* 285:446.
- 19.—Staehelin.T., B.Duner, J.Schmidt, B.Takacs, J.Stocker, V. Miggiano, C.Stähli, M.Rubinstein,  
W.P.Levy, R.Hershberg, and S.Pestka. 1981. "Production of hybridomas secreting monoclonal  
25 antibodies to the human leukocyte interferons". *Proc.Natl.Acad.Sci.USA* 78:1848. 25
- 20.—Imai,M., T.Sano, Y.Yanase, K.Miyamoto, S.Yonehara, H.Mori, T.Honda, S.Fukuda, T.Naka-  
mura, Y.Miyakawa, and M.Mayumi. 1982. "Demonstration of two subtypes of human leukocyte  
interferon (IFN $\alpha$ ) by monoclonal antibodies". *J.Immunol.* 128:2824.
- 21.—Novick,D., Z.Eshhar, and M.Rubinstein. 1982. "Monoclonal antibodies to human  $\alpha$ -inter-  
30 feron and their use for affinity chromatography". *J.Immunol.* 129:2244. 30
- 22.—Shearer,M., J.Taylor-Papadimitrou, D.Griffin, and F.Balkwill. 1984. "Monoclonal antibodies  
that distinguish between subspecies of human interferon- $\alpha$  and that detect interferon oligomers".  
*J.Immunol.* 133:3096.
- 23.—Lydon,N.B., C.Favre, S.Bove, O.Neyret, S.Benureau, A.M.Levine, G.F.Seelig, T.L. Naga-  
35 bhushan, and P.P.Trotta. 1985. "Immunochemical mapping of  $\alpha$ -2 interferon" *Biochemistry 35*  
24:4131.
- 24.—Staehelin,T., C.Stähli, D.S.Hobbs, and S.Pestka. 1981. "A rapid quantitative assay of high  
sensitivity for human leukocyte interferon with monoclonal antibodies". *Methods Enzymol.*  
79:589.
- 25.—Allen,G., K.H.Fantes, D.C.Burke, and J.Morser. 1982. "Analysis and purification of human  
40 lymphoblastoid (Namalwa) interferon using a monoclonal antibody", *J.Gen.Virol.* 63:207. 40
- 26.—Staehelin,T., D.S.Hobbs, H—F.Kung, and S.Pestka. 1981. "Purification of recominant hu-  
man leukocyte interferon (IFLrA) with monoclonal antibodies". *Methods Enzymol.* 79:505.
- 27.—Galfré,G., and C.Milstein. 1981. "Preparation of monoclonal antibodies: strategies and  
45 procedures". *Methods Enzymol.* 73:3. 45
- 28.—Sánchez-Madrid,F., P.Szklut, and T.A.Springer. 1983. "Stable hamster-mouse hybridomas  
producing IgG and IgM hamster monoclonal antibodies of defined specificity". *J.Immunol.*  
130:309.
- 29.—Ey,P.L., S.J.Prowse, and C.R.Jenkin. 1978. "Isolation of pure IgG1, IgG2a, and IgG2b  
50 immunoglobulins from mouse serum using protein A-Sepharose". *Immunochemistry.* 15: 429. 50
- 30.—Fraker,P.J., and J.C.Speck. 1978. "Protein and cell membrane iodinations with a sparingly  
soluble chloroamide 1,3,4,6-tetrachloro-3 $\alpha$ ,6 $\alpha$ -diphenyl glycoluril". *Biochem. Biophys.Res.Com-  
mun.* 80:849.
- 31.—Sánchez-Madrid,F., D.Davignon, E.Martz, and T.A.Springer. 1982. "Antigens involved in  
55 mouse cytotoxic T-lymphocyte (CTL)-mediated killing: functional screening and topographic rela- 55  
tionship". *Cell.Immunol.* 73:1.
- 32.—Ware,C.F., J.L.Reade, and D.L.Der. 1984. "A rat anti-mouse kappa chain specific mono-  
clonal antibody, 187.1.10; purification, immunochemical properties and its utility as a general  
second-antibody reagent", *J.Immunol.Methods* 74:93.
- 33.—Muñoz,A., L.Carrasco, and M.Fresno. 1983. "Enhancement of susceptibility of HSV—1  
60 infected cells to natural killer lysis by interferon", *J.Immunol.* 131:783. 60
- 34.—Landergrén,V. 1984. "Measurement of cell number using an endogenous enzyme, hexo-  
saminidase: Applications for the detection of lymphokines and cell surface antigens". *J.Immunol.-  
Methods* 67:379.
- 35.—De Landázuri,M.O., M.López-Botet, T.Timonen, J.Ortaldo, and R.B.Herberman. 1981. "Hu- 65  
65

man large granular lymphocytes: Spontaneous and interferon boosted NK activity against adherent and nonadherent tumor cell lines", J. Immunol. 127:1380.

36.-Zoon,K.C., and H.Arnheiter. 1984. "Studies of the interferon receptors". Pharmac. Ther. 24:259.

5 37. Branca,A.A., C.R.Faltynek, S.B.D'Alessandro, and C.Baglioni. 1982. "Interaction of interferon with cellular receptors", J.Biol.Chem. 257:13291. 5

38.-Raziuddin,A., F.H.Sarkar, R.Dutkowski, L.Shulman, F.H.Ruddle, and S.Gupta. 1984. "Receptors for human  $\alpha$  and  $\beta$  interferon but not for  $\gamma$  interferon are specified by human chromosome 21". Proc.Natl.Acad.Sci.USA 81:5504.

10 39.-Joshi,A.R., F.H.Sarkar, R.Dutkowski, and S.L.Gupta. 1982. "Interferon receptors-Cross-linking of human leukocyte interferon  $\alpha$ -2 to its receptor on human cells". J.Biol.Chem. 257:13884. 10

40.-Whittall,J.T.D., R.M.King, and D.C.Burke. 1984. "The reaction of the anti-interferon- $\alpha$  monoclonal antibody, NK2, with different interferons", J.Gen.Virol. 65:629.

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#### CLAIMS

1. A monoclonal antibody to IFN which inhibits the cell-binding capability of the IFN or which inhibits the cytotoxicity-boosting activity of the IFN mediated by NK cells.

2. An antibody according to claim 1 to IFN  $\alpha$ .

20 3. An antibody according to claim 2 to rIFN $\alpha$ -2. 20

4. A method of preparing an antibody according to any of claims 1 to 3 by raising anti-IFN monoclonal antibodies by techniques known *per se* and screening them for the desired inhibitor activity, then culturing the cell line which produces the selected monoclonal antibody.

5. An antibody to IFN obtained by a method according to any of claims 1 to 4.